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Differential acute effects of sleep on spontaneous and stimulated production of tumor necrosis factor in men

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ABSTRACT

Tumor necrosis factor (TNF) is considered a key molecule in the regulation of sleep in health and disease. Conversely, sleep compared to sleep deprivation can modulate TNF release, but overall results are conflicting. In this study we focused on the influence of sleep on spontaneous, i.e., unstimulated TNF production, which might be involved in sleep regulation under normal non-infectious conditions, and on lipopolysaccharide (LPS)-stimulated TNF production, which reflects the capacity of the immune system to respond to a pathogen. To this end, we monitored 10 healthy men during a regular sleep-wake cycle and during 24 h of wakefulness while blood was sampled repeatedly to analyze circulating TNF levels in serum as well as intracellular TNF production in monocytes spontaneously and after stimulation with LPS employing whole blood cell cultures. In addition we assessed numbers of monocyte subsets and levels of various hormones in blood. In comparison with nocturnal wakefulness, sleep acutely decreased serum TNF levels, with no parallel decrease in spontaneous monocytic TNF production, but was associated with a striking nighttime increase in the percentage of TNF producing monocytes after stimulation with LPS. The following day circulating TNF showed a reverse pattern with higher levels after regular sleep than after the nocturnal vigil. The mechanisms mediating the differential effects of sleep on circulating TNF (acutely decreased) vs. stimulated monocytic TNF production (acutely increased) remain unclear, although explorative correlational analyses pointed to a regulatory involvement of cortisol, norepinephrine and prolactin. The acute enhancing effect of sleep on LPS stimulated monocytic TNF production adds to the notion that nocturnal sleep favors immune defense to a microbial challenge.

1. Introduction

In the bidirectional crosstalk between sleep and the immune system, tumor necrosis factor (TNF) is a key signal. TNF, together with other cytokines such as interleukin (IL)-1 is a 'sleep regulatory substance' and has a role in the homeostatic regulation of sleep in normal physiological conditions (Bryant and Curtis, 2013; Krueger et al., 1999). Thus, neuronal activity during wakefulness triggers the release of TNF, which in turn promotes non-rapid eye movement (NREM) sleep and enhances slow wave activity (Churchill et al., 2008; Krueger et al., 2008). In accordance with its sleep regulating function, brain levels of endogenous TNF fluctuate with the sleep-wake cycle with a peak during the early resting time and a trough after elapsed sleep (Bredow et al., 1997; Cearley et al., 2003; Floyd and Krueger, 1997; Krueger et al., 1999). Moreover, during experimental sleep deprivation, spontaneous TNF production in the brain is increased (Taishi et al., 1999; Zielinski et al., 2014). However, this pattern characteristic for a sleep regulatory substance has not been obtained in human studies measuring circulating TNF levels in blood: 24 h observations revealed either no overt rhythm (Gudewill et al., 1992; Togo et al., 2009; Vgontzas et al., 2004) or a morning peak in levels of TNF (Vgontzas et al., 2002, 2003). In sleep deprivation experiments in healthy subjects circulating TNF remained unchanged (Irwin et al., 2004; Lekander et al., 2013; Ruiz et al., 2012; Shearer et al., 2001; Vgontzas et al., 2007) or increased only at certain times after prolonged periods of sleep loss, i.e., in the afternoon after 34 h of total sleep

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deprivation (Chennaoui et al., 2011) or in the morning after 7 days of sleep restriction (Chennaoui et al., 2011; Vgontzas et al., 2004). However, chronic sleep disturbances were indeed found to be associated with robust increases in circulating TNF levels, in particular during the daytime period, in patients with insomnia (Vgontzas et al., 2002) and sleep apnea (Minoguchi et al., 2004; Nadeem et al., 2013), as well as in habitual short sleepers (Patel et al., 2009) and in pregnant women (Okun and Coussons-Read, 2007). Along with increases in circulating levels of IL-1, IL-6 and C-reactive protein (CRP) these signs of low-grade systemic inflammation have been proposed to explain the observed connection between sleep debt and an increased risk for inflammatory diseases (Ananthakrishnan et al., 2013; Grandner et al., 2013; Irwin, 2014; Kinnucan et al., 2013; Mullington et al., 2009; Seay et al., 2013).

Apart from these subtle but persisting increases in TNF during low-grade systemic inflammation, high amounts of TNF are produced chiefly by activated macrophages and monocytes upon an infectious challenge. As such TNF serves the acute regulation of local and systemic inflammation and, thus, the efficient eradication of pathogens. TNF boosts innate and adaptive immune responses to bacterial and viral infections through multiple mechanisms: it enhances (i) antigen-presenting cell activation, migration and maturation (Banchereau et al., 2000; Kaisho et al., 2001), (ii) phagocytosis (Hess et al., 2009), (iii) prostaglandin production (Baud et al., 1992), (iv) the acute-phase response (Gabay and Kushner, 1999), (v) T cell proliferation (Cope et al., 1994), and (vi) its own production via positive autocrine feedback mechanism (Blasi et al., 1994; Imamura et al., 1987). Employing stimulation with the toll-like receptor (TLR)2/TLR4 ligand LPS several human studies revealed a circadian rhythm in the production of TNF that peaks during the night (Entzian et al., 1996; Hermann et al., 2006; Petrovsky et al., 1998; Zabel et al., 1993). The contribution of sleep to this rhythm, however, is presently obscure as respective studies provided rather mixed outcomes with enhancing (Weil et al., 2009), suppressing (Uthgenannt et al., 1995), and no (Ashley et al., 2013; Born et al., 1997; Haack et al., 2001) effects of sleep on LPS-stimulated TNF production.

Against this backdrop in the present experiments we aimed to assess in humans the impact of sleep on both spontaneous and stimulated production of TNF by monocytes as well as on levels of TNF circulating in blood. Based on previous reports in sleep apnea patients (Minoguchi et al., 2004), we expected parallel changes in circulating levels of TNF and spontaneous production of TNF by monocytes, although the latter are only one of the sources of TNF in the body. We hypothesized that in our healthy subjects, both measures display the temporal pattern typical for a sleep regulatory substance, acutely decreasing across nocturnal sleep and increasing during subsequent wakefulness. In contrast, given the supportive function of sleep for host defense (Lange et al., 2011), for LPS-stimulated production of TNF we anticipated an acute sleep-dependent increase specifically during nighttime. In order to cover the possibility that effects of sleep on cytokine production are restricted to certain time intervals (Chennaoui et al., 2011), or even are opposite in direction during night and daytime (Redwine et al., 2000; Vgontzas et al., 1999), we performed repeated blood draws covering an entire 24-h period. In addition, to control for potential confounding influences of cell composition (Born et al., 1997) we analyzed TNF production on the single cell level in monocytes by flow cytometry and measured also the proportion of two monocyte subsets which are known to differ both in their capacity to produce TNF and in their regulation by sleep and the circadian system (Dimitrov et al., 2007, 2013; Nguyen et al., 2013; Ziegler-Heitbrock et al., 2010).

Because the effect of sleep on the immune system is believed to be caused by sleep-associated changes in immunoregulatory hormones such as growth hormone (GH), prolactin, testosterone, cortisol, epinephrine, and norepinephrine, we also measured these hormones in blood and performed supplementary in vitro experiments to elucidate their role in mediating the influence of sleep on LPS stimulated TNF production (Besedovsky et al., 2012; Bouman et al., 2005; Cipollaro de et al., 1998; Petrovsky, 2001; Severn et al., 1992).

2. Materials and methods

2.1. Participants

Ten physically and mentally healthy men were included in the study (mean age 25.3, range 18–30 years, mean body mass index 23.5, range 20–26 kg/m^2) from a larger parent trial that investigated sleep, circadian rhythm, and immune parameters such as IL-6, IL-12, and blood cells counts (Dimitrov et al., 2007, 2009). They were non-smokers, did not suffer from sleep disturbances, and were not taking any medication at the time of the experiments. None had a medical history of any relevant chronic disease or mental disorder. Acute illness was excluded by physical examination and routine laboratory investigation, including chemistry panel, CRP concentration <6 mg/L, and a white blood cell (WBC) count <9000/μL. Women were not included in the study to keep the subject sample most homogenous with regard to sleep and endocrine parameters.

The men were synchronized by daily activities and nocturnal rest. They had a regular sleep-wake rhythm for at least 6 weeks before the experiments. During the week preceding the study, they were required to turn off lights for nocturnal sleep between 11 pm and 11:30 pm, to get up by 7 am the next morning, and not to take any naps during the day. Adherence to these instructions was exclusively confirmed by questionnaires. All subjects spent one adaptation night in the laboratory in order to become accustomed to the experimental setting. The presence of signs of sleep disturbances, including apnea and nocturnal myoclonus, was excluded by interview and by recordings during this night. The study was approved by the Ethics Committee of the University of Lübeck, and all participants gave written informed consent.

2.2. Procedure

Experiments were performed according to a within-subject cross-over design. Each man participated in two experimental conditions, each starting at 8 pm and ending 24 h later. One condition ('sleep') included a regular sleep-wake cycle whereas in the other condition ('wake') subjects remained awake throughout the 24-h experimental period. Both experimental sessions for a subject were separated by at least four weeks, and the order of conditions was balanced across subjects. In the 'sleep' condition, sleep was allowed between 11 pm (lights off) and 7 am in the morning. On the 'wake' condition, subjects stayed awake in bed in a half-supine position between 11 pm and 7 am. During this time they were watching TV, listening to music and talking to the experimenter at normal room light (about 300 lux).

On both conditions, blood was sampled first at 8 pm, then every 1.5 h between 11 pm and 8 am, and every 3 h between 8 am and 8 pm the next day. Blood was sampled via an intravenous forearm catheter which was connected to a long thin tube and enabled blood collection from an adjacent room without disturbing the subject's sleep. To prevent clotting, about 700 mL of saline solution were infused throughout the 24-h experimental period. The total volume of blood sampled during a session was 250 ml. Blood samples were always processed immediately after sampling.

Sleep and sleep stages were determined off-line from polysomnographic recordings following standard criteria

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(Rechtschaffen and Kales, 1968). For each night, sleep onset (with reference to lights off at 11 pm), total sleep time, and the time as well as the percentage of total sleep time spent in the different sleep stages (wake, NREM sleep stages 1, 2, 3, and 4, slow-wave sleep (SWS, equal to the sum of stage 3 and 4), and rapid eye movement (REM) sleep) were determined.

2.3. Monocyte subpopulations

Absolute counts of $CD45^+CD14^{+/dim}HLA\text{-}DR^+$ total monocytes, as well as its $CD14^{+/dim}CD16^+$ (intermediate plus non-classical), and $CD14^+CD16^-$ (classical) subsets were determined by a 'lyse no wash' flow cytometry procedure as previously reported (Dimitrov et al., 2007).

2.4. Spontaneous and LPS-stimulated monocytic intracellular TNF production by flow cytometry

Whole blood was analyzed for spontaneous and LPS-stimulated intracellular monocytic TNF production. Two concentrations of LPS were used – 100 and 300 pg/ml – that are in the range of endotoxin levels found previously in infections (Matsumoto et al., 1991; Wiedermann et al., 1999), and were determined to be appropriate for significant activation of monocytes in preliminary experiments with 10 to 90% of the monocytes producing TNF. Blood cells were incubated in sterile polypropylene tubes with or without LPS (E. coli 0111:B4, catalog # L4391, Sigma–Aldrich, St. Louis, MO) for 6 h at 37 °C with 5% CO_2. To stop cytokine excretion (allowing intracellular detection), brefeldin A (10 μg/mL, Sigma–Aldrich) was added for the last 4 h of LPS incubation.

Intracellular TNF production of monocytes was evaluated by multiparametric flow cytometry using fluorochrome-conjugated antibodies. First, erythrocytes were lysed using ammonium chloride solution followed by centrifugation (5 min at 500×*g*). The cell pellet was washed once with PBS, containing 0.1% azide and 0.5% bovine serum albumin, prior to incubation with monoclonal antibodies (15 min) for the monocytes identification: anti-CD14/FITC, and anti-HLA-DR/PE (BD Biosciences). Because LPS can lead to significant downregulation of CD16 expression, we did not include this surface marker in our ex vivo assay and thus measured TNF production in total monocytes (Dimitrov et al., 2013). After fixation and permeabilization according to the manufacturer's instructions (Cytofix/Cytoperm Kit, BD Biosciences), cells were stained intracellularly with TNF/APC antibody (BD Biosciences). Monocytes were distinguished from lymphocytes and granulocytes by means of their forward and side scatter (FSC and SSC) characteristics and were identified as $CD14^{+/dim}HLA\text{-}DR^+$ cells. At least 10,000 $CD14^{+/dim}HLA\text{-}DR^+$ monocytes were collected for each tube on a dual-laser FACSCalibur (BD Biosciences), and subsequently analyzed for TNF expression using commercially available software (CellQuest; BD Biosciences). The percentage of the $CD14^{+/dim}HLA\text{-}DR^+$ cells that were positive for TNF ('TNF^+ monocytes') was assessed in unstimulated and in LPS-stimulated cells, with the former representing a measure of spontaneous TNF production.

2.5. Measurement of circulating TNF and hormone levels

Samples for measuring circulating TNF and hormone concentrations were kept frozen at −70 °C until assay. TNF was measured in serum using a high-sensitivity ELISA kit (Cat. # HSTA00D, R&D Systems, Minneapolis, Minn). GH, prolactin, cortisol, and testosterone were measured in serum using commercial assays (Immulite, DPC-Biermann GmbH, Bad Nauheim, Germany). Epinephrine and norepinephrine were measured in plasma by standard high-performance liquid chromatography. Sensitivity, and intraassay and interassay coefficients of variation were as follows: TNF 0.2 pg/ml, less than 10.4%. GH 0.01 ng/ml, less than 6.6%, prolactin 0.16 ng/ml, less than 9.5%, cortisol 0.2 μg/dl, less than 10%; testosterone 10 ng/dl, less than 13.7%; epinephrine 2.0 pg/ml, less than 5.6%; norepinephrine 5.0 pg/mL, less than 6.1%; All measurements were above the sensitivity levels and thereby detectable.

2.6. In vitro testing of hormonal mediators

Prolactin, cortisol and norepinephrine were candidate hormonal mediators of temporal changes in LPS-stimulated TNF production as revealed by correlation analyses in the main experiment. To test the influence of changes in these hormones during nocturnal sleep on the TNF production we performed supplementary in vitro experiments in additional 12 men with blood drawn at 1 am during regular sleep. Subject characteristics and procedures were the same as in the main experiment. Percentages of TNF^+ monocytes were determined after preincubation for 10 min at room temperature of whole blood with isotype antibody (R&D Systems), prolactin antibody (1 μg/mL anti-human prolactin antibody; R&D Systems), cortisol (4 and 20 μg/dl; Sigma–Aldrich) or norepinephrine (170 and 510 pg/ml; Sigma–Aldrich). Further stimulation (with 300 pg/ml LPS) and staining protocols were identical to those in the main experiment.

2.7. Statistical analysis

Data are presented as means ± SEM. Statistical analyses were generally based on repeated measures ANOVA with subsequent post hoc contrasts. ANOVA factors were either 'condition' (sleep/wake) and 'time' (single time points) or 'condition' (sleep/wake) and 'period' (night/day, using average levels across the five time points during night and day, respectively). Whenever appropriate, non-significant differences in baseline measures (i.e., at 8 pm and 11 pm) between conditions were used as covariates to correct for day-to-day variations in hormones and immune parameters. Degrees of freedom were corrected using the Greenhouse–Geisser procedure. To identify significant rhythms, cosinor analysis was performed separately for the 'wake' and 'sleep' condition using Chronolab employing a period of 12 or 24 h (Mojon et al., 1992). To assess relationships between rhythms in TNF measures and diurnal variations of monocyte subsets and hormonal concentrations, univariate Pearson's correlation analyses were performed. For these analyses, Pearson's coefficients between the different parameters were calculated for each subject and condition (sleep vs. wake). The single subject coefficients were *z*-transformed and *t*-test significances (comparison to '0') for the whole group of subjects were calculated separately for both conditions. We present mean correlation coefficients and applied Bonferroni correction yielding a significance level of $p < .0025$ (.05/10/2 (10 tested parameters, 2 conditions). Missing single values were replaced by linear interpolation which happened in less than 2% of the data.

3. Results

3.1. Serum TNF levels

Compared with the 'wake' condition, sleep seemed to acutely decrease nocturnal levels of TNF in serum, while during the following day TNF concentrations appeared to be increased (Fig. 1). These differences failed to reach significance in the ANOVA that included single time points ($p \geqslant .068$ for condition effect or for condition × time interaction). However, average TNF levels in the 'sleep' condition were lower at night and higher during the day compared to the 'wake' condition ($F(1,9) = 9.7$; $p = .01$ for condition × period interaction; see insert of Fig. 1 for post hoc comparisons). The

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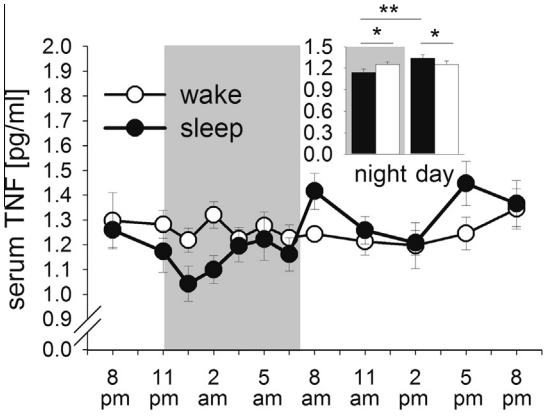

Fig. 1. Sleep acutely reduces serum levels of tumor necrosis factor (TNF). Mean ± SEM serum TNF concentrations are shown during a regular sleep-wake cycle (sleep, filled circles) and continuous wakefulness (wake, open circles). The shaded area depicts bed time. Bar graphs (inserts) indicate mean ± SEM values for nighttime (= bedtime) and daytime. Significance is indicated for pairwise comparison between conditions. $n = 10$, $^{*}p < .05$, $^{**}p < .01$.

reducing effect of sleep on circulating TNF seemed to be restricted to the early night half. Comparing TNF levels during early (0:30 – 2 am) and late nocturnal sleep (3:30 – 7 am) indeed indicated that during the sleep period TNF levels were increasing ($p > .02$) rather than further decreasing. The mean 24-h TNF secretion was not different between the 'wake' and 'sleep' conditions ($p > .7$ for condition effect).

Cosinor analysis revealed 12 and 24 h rhythms in serum TNF concentrations only during the regular sleep-wake cycle. Whereas the 24-h rhythm failed to reach significance ($p = .07$), the 12 h rhythm was highly significant ($p = .003$), with the fitted cosine curve showing a mesor of 1.23 ± 0.05 pg/ml, an amplitude of 0.1 ± 0.01 pg/ml, and an acrophase at 7:07 am/pm ± 17 min.

3.2. Spontaneous and LPS-stimulated TNF production by monocytes

The percentage of monocytes spontaneously producing TNF (i.e., without LPS stimulation) accounted for only ~1% of total monocytes and appeared to be decreased at 3 am in the 'sleep' compared to the 'wake' condition (Fig. 2A). However, this difference was not evident in the ANOVA ($p > .5$ for condition × time interaction, for condition × period interaction, or for condition effect). Cosinor analysis showed a lack of a significant 12 or 24 h rhythm during both conditions ($p = .7$).

As expected stimulation with 100 and 300 pg/ml LPS increased the percentage of TNF^+ monocytes to about 25% and 45% of total monocytes, respectively (Rontgen et al., 2004). Compared with the 'wake' condition, sleep strikingly increased the percentage of LPS-stimulated TNF^+ monocytes. The rise extended over the whole nocturnal sleep period, peaked at about 2 am and was completely blocked when subjects remained awake at night. Nocturnal differences between the 'sleep' and 'wake' conditions reached significance in the ANOVA only with the 300 pg/ml LPS dose ($F(1,8) = 1.5$; $p = .26$ and $F(1,8) = 9.9$; $p = .014$ for condition effect with 100 and 300 pg/ml LPS, respectively; ANOVA factors were condition and time, see Fig. 2C for post hoc comparisons at single time points). Cosinor analysis confirmed a significant rhythm in TNF^+ monocytes after stimulation with 100 and 300 pg/ml LPS only during the regular sleep-wake cycle ($p < .01$ for both concentrations of LPS), with the fitted cosine curve showing a mesor (mean level) of 25.9 ± 1.2% cells, a (peak-to-trough) amplitude of 8.4 ± 1.7%, and an acrophase (peak time) at 1:49 am ± 26 min with 100 pg/ml LPS, and a mesor of 48.8 ± 2.7% cells, an amplitude of

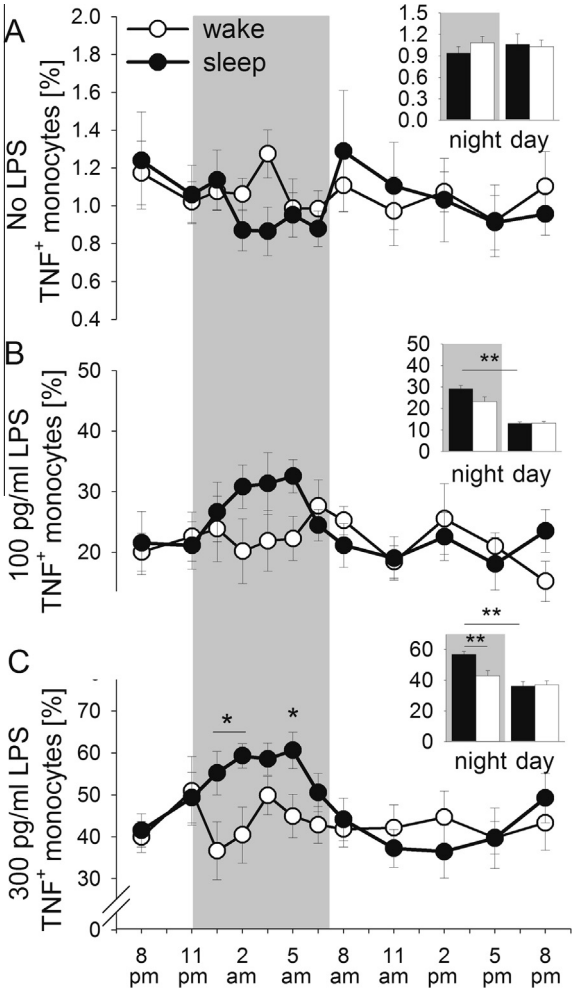

Fig. 2. Sleep enhances stimulated production of tumor necrosis factor (TNF) by monocytes. Mean ± SEM percentages of TNF-producing monocytes (with reference to total monocytes) without stimulation (A, no lipopolysaccharide (LPS) = spontaneous TNF production) and after stimulation with 100 pg/ml LPS (B) and 300 pg/ml LPS (C) during a regular sleep-wake cycle (sleep, filled circles) and continuous wakefulness (wake, open circles). The shaded area depicts bed time. Bar graphs (inserts) indicate mean ± SEM values for nighttime (= bedtime) and daytime. Significance is indicated for pairwise comparison between conditions. $n = 10$, $^{*}p < .05$, $^{**}p < .01$.

11.9 ± 1.7%, and an acrophase at 1:59 am ± 28 min with 300 pg/ml LPS. Accordingly, TNF^+ monocytes significantly changed from night to day ($F(1,8) = 11.4$; $p = .01$ and $F(1,8) = 14.2$; $p < .01$ for period effect with 100 and 300 pg/ml LPS, respectively), but in post hoc comparisons these differences were evident only in the 'sleep' condition (see inserts of Fig. 2B and C).

3.3. Monocyte subpopulations and their associations with TNF production by monocytes

Monocytes can be subdivided into a smaller $CD16^+$ subpopulation (consisting of intermediate and nonclassic monocytes) and a larger $CD14^+CD16^-$ subpopulation (classical monocytes). As previously published for an extended subject sample (Dimitrov et al., 2007), sleep compared with nocturnal wakefulness distinctly decreased the number of circulating $CD16^+$ monocytes and, thus, induced a significant circadian rhythm with an acrophase at 19:26 pm ($p = .01$; $F(11,88) = 2.6$; $p = .05$ for condition × time interaction; Fig. 3A). In contrast to $CD16^+$ monocytes, classical $CD14^+CD16^-$ monocytes were not influenced by sleep

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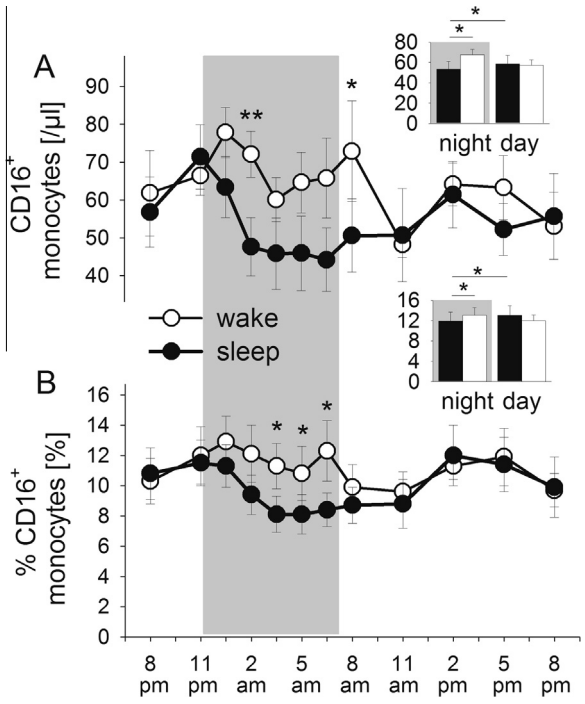

Fig. 3. Sleep decreases numbers and percentages of circulating CD16$^+$ monocytes. Mean ± SEM CD16$^+$ monocyte absolute counts (A) and their proportion of total monocytes (B) during a regular sleep-wake cycle (sleep, filled circles) and continuous wakefulness (wake, open circles). The shaded area indicates bed time. Bar graphs (inserts) indicate mean ± SEM values for nighttime (= bedtime) and daytime. Significance is indicated for pairwise comparison between conditions. $n = 10$, $^*p < .05$; $^{**}p < .01$.

Table 1
Correlation analyses.

	Serum TNF	% TNF$^+$ monocytes		
		No LPS	100 pg/ml LPS	300 pg/ml LPS
% CD16$^+$ monocytes	n.s.	n.s.	n.s.	n.s.
Hormones				
Growth hormone	n.s.	n.s.	.28$^{\#}$	n.s.
Prolactin	n.s.	n.s.	.33$^{\#\#}$	.41$^{\#\#}$
Testosterone	n.s.	n.s.	.41$^{\#\#}$	n.s.
Cortisol	n.s.	n.s.	−.33$^{\#}$	−.38$^{\#}$
Norepinephrine	.38$^{\#\#}$	n.s.	−.46$^{\#\#}$	−.45$^{\#\#}$
Epinephrine	n.s.	n.s.	n.s.	n.s.
% TNF$^+$ monocytes				
No LPS	n.s.			
100 pg/ml LPS	n.s.	n.s.		
300 pg/ml LPS	−.33$^{\#}$	n.s.	.71$^{\#\#}$	

Pearson coefficients derived from correlation analyses between temporal variations in serum TNF, in the percentage of TNF$^+$ monocytes, in the proportion of the CD16$^+$ subset in the total pool of monocytes (% CD16$^+$), and in hormonal levels during the 'sleep' condition. No significant correlations were evident in the 'wake' condition. Bonferroni correction was applied yielding a significance level of $p < .0025$ (0.05/10/2 (10 tested parameters, 2 conditions)). TNF production was measured after culture of whole blood cells without (no LPS, spontaneous TNF production) or in the presence of LPS (100 or 300 pg/ml, stimulated TNF production) for 6 h. $^{\#}p < .0025$; $^{\#\#}p < .001$; n.s., not significant.

(509.1 ± 89.4 vs. 425.3 ± 59.9 for the nighttime period of 'wake' vs. 'sleep' conditions; $p > .12$, for respective main and interaction effects; data not shown) and failed to show a significant rhythm in the 'sleep' or 'wake' condition ($p > .3$).

The selective drop of CD16$^+$ monocyte counts during nocturnal sleep, with unchanged CD14$^+$CD16$^-$ counts, led to an imbalance between these populations with respect to total monocytes, i.e., the proportion of the CD16$^+$ subset in the total pool of monocytes (% CD16$^+$) was distinctly lowered during nocturnal sleep compared to continuous wakefulness ($F(11,88) = 2.7$; $p = .04$ for condition × time interaction; Fig. 3B), and followed a rhythm that paralleled the absolute CD16$^+$ monocyte counts with an acrophase at 18:20 pm ($p = .02$).

Given that CD16$^+$ monocytes produce a larger amount of TNF spontaneously and upon LPS stimulation than CD14$^+$ cells (Dimitrov et al., 2013; Selkirk et al., 2009), we wondered whether the sleep-associated changes in the proportion of the monocyte subsets might have confounded the effects of sleep on TNF measures. However, Pearson correlational tests did not identify % CD16$^+$ monocytes as a significant predictor of circulating TNF levels and spontaneous or LPS-stimulated TNF production by monocytes (Table 1).

3.4. Hormones and their association with TNF measures

Compared with continuous wakefulness, regular sleep was accompanied by the typical changes in endocrine activity (summarized in Fig. 4). Concentrations of GH were increased selectively during early sleep, between 0:30 am and 2 am ($F(11,99) = 6.5$; $p < .01$, for condition × time interaction, see Fig. 4 for comparisons at single time points). Plasma concentrations of prolactin were strongly enhanced during sleep throughout the night, compared with the 'wake' condition ($F(11,99) = 7.7$; $p < .001$, for condition × time interaction), whereas testosterone levels were selectively increased during late sleep ($F(11,99) = 3.7$; $p < .05$, for condition × time interaction). Cortisol and norepinephrine concentrations appeared to be reduced during sleep time, but the difference between 'sleep' and 'wake' conditions did not reach significance in the ANOVA ($p = .1$ and .24, for condition effect for cortisol and norepinephrine, respectively). Epinephrine levels were significantly decreased during sleep at 0:30 am ($F(1,9) = 5.0$; $p = .05$, for condition effect). Whereas prolactin, cortisol and norepinephrine followed a significant rhythm in both conditions, growth hormone, testosterone and epinephrine showed pronounced rhythms in the 'sleep' condition only ($p < .05$).

To explore whether TNF measures were linked to each other and to temporal changes in hormones, we calculated single correlations between individual serum TNF levels, percentages of TNF$^+$ monocytes, and hormonal levels of both the 'sleep' and 'wake' condition. No correlations were evident in the 'wake' condition. In the 'sleep' condition serum TNF levels correlated positively with norepinephrine and negatively with TNF$^+$ monocytes stimulated with 300 pg/ml LPS (Table 1). The percentage of monocytes spontaneously producing TNF did not correlate with serum TNF levels ($r = .03$, $p = .7$) or with the other parameters (Table 1). TNF$^+$ monocytes correlated positively with prolactin, and negatively with cortisol and norepinephrine for both LPS doses (Table 1). Additional positive correlations with GH and testosterone were observed only for TNF$^+$ monocytes stimulated with 100 pg/ml LPS. Correlation analyses yielded comparable results, when a lag time of 3 h was introduced between cortisol and TNF measures (data not shown).

3.5. Sleep

Polysomnographic recordings ensured that sleep was typical for laboratory conditions. Mean (± SEM) sleep onset latency was 24.1 ± 4.6 min. Mean values were for sleep time, 435.8 ± 16.5 min; time in stage 1 sleep, 29.8 ± 7.9 min; stage 2 sleep, 237.4 ± 18.6 min; SWS, 74.6 ± 6.6 min; and REM sleep, 74.4 ± 7.1 min.

3.6. Effects of hormones on LPS-stimulated production by monocytes

To further explore a possible contribution of sleep-associated hormonal changes to the regulation of LPS-stimulated TNF production by monocytes, we performed supplementary in vitro

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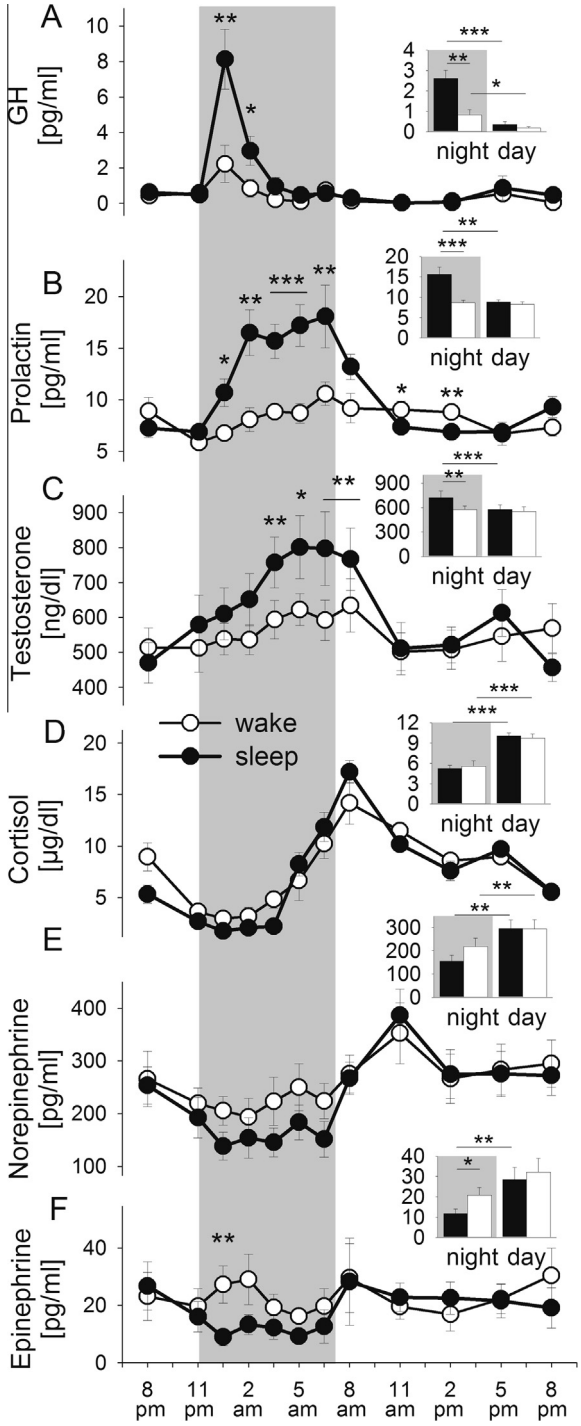

Fig. 4. Sleep impacts hormonal levels. Mean ± SEM hormone concentrations of growth hormone (GH) (A), prolactin (B), testosterone (C), cortisol (D), norepinephrine (E), and epinephrine (F) during a regular sleep-wake cycle (sleep, filled circles) and continuous wakefulness (wake, open circles). The shaded area indicates bed time. Bar graphs (inserts) indicate mean ± SEM values for nighttime (= bedtime) and daytime. Significance is indicated for pairwise comparison between conditions. $n = 10$, $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

experiments in which blood samples were drawn from additional sleeping subjects at 1 am and stimulated with 300 pg/ml LPS. We then determined numbers of TNF^{+} monocytes after adding prolactin antibodies to block peak levels of prolactin, as well as after adding cortisol or norepinephrine in two different concentrations to increase endogenous nadir levels of these stress hormones to levels that are normally observed during nocturnal wakefulness and

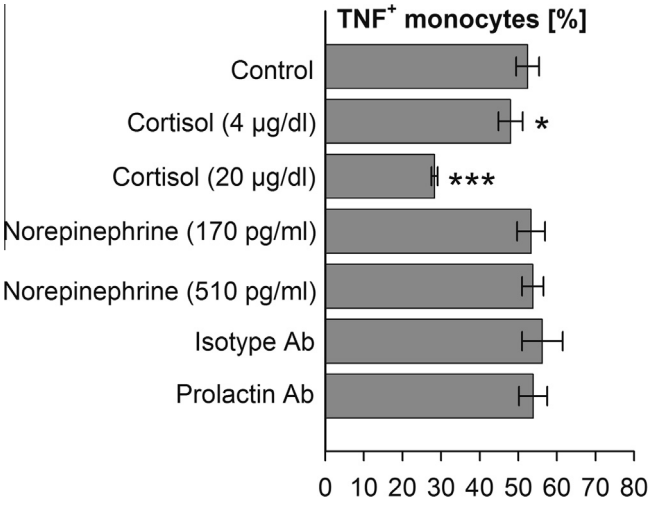

Fig. 5. Cortisol reduces stimulated tumor necrosis factor (TNF) production by monocytes in vitro. Mean ± SEM percentages of TNF^{+} monocytes after stimulation of whole blood sampled at 1 am from 12 sleeping men with 300 pg/ml lipopolysaccharide (LPS) in the presence of cortisol, norepinephrine, isotype antibody (Ab, isotype matched control of anti-prolactin antibody) and prolactin Ab. Control values refer to untreated samples. Significance is indicated for pairwise comparisons with control values. $^{*}p < .05$; $^{***}p < .001$.

daytime activity, respectively. We only found a reduction in the percentage of TNF^{+} monocytes after application of cortisol at concentrations of 4 µg/dl ($p < .05$) and 20 µg/dl ($p < .001$) (Fig. 5).

4. Discussion

The present experiments aimed at elucidating the impact of sleep on spontaneous and LPS-stimulated TNF production by monocytes, as well as on circulating blood levels of TNF in healthy humans. Sleep in comparison with nocturnal wakefulness acutely reduced circulating TNF levels. However, the reduction was confined to the early nocturnal sleep period. Thereafter levels were increasing toward the end of the night and, on the following day, were even higher than during continuous wakefulness. Also, sleep did not significantly affect spontaneous monocytic TNF production. In contrast to its acute reducing effect on serum TNF levels, sleep produced a striking increase in the percentage of LPS-stimulated TNF^{+} monocytes at night. Exploratory analyses indicated that the decrease in serum TNF levels and the increase in LPS-stimulated monocytic TNF production during nocturnal sleep were not confounded by changes in monocyte subsets, but were related to hormonal changes along the sleep-wake cycle.

4.1. TNF levels in blood and unstimulated TNF production by monocytes

To the best of our knowledge, this is the first report in humans showing an acute decrease in circulating TNF levels during sleep. This pattern was previously revealed for TNF brain levels in animals, which substantiated the view of TNF being a sleep regulatory substance (Krueger et al., 1999; Zielinski et al., 2014). The difference in serum TNF levels between early sleep and nocturnal wakefulness was ~0.2 pg/ml, which appears to be of considerable magnitude for an acute sleep manipulation of only few hours in healthy subjects, when compared to chronic clinical conditions like in obese subjects and sleep apnea patients with increments in circulating TNF of ~0.5 and ~1.0 pg/ml, respectively, with reference to healthy controls (Minoguchi et al., 2004). While the drop in TNF levels during nocturnal sleep is essentially consistent with a

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sleep regulatory role, the decrease appeared to be restricted to the early part of sleep dominated by SWS. During the remaining period of sleep, TNF levels were increasing, rather than decreasing, and reached a first peak in the morning after awakening. At this time TNF levels were even higher after regular sleep than after the nocturnal vigil. In sum, apart from its dynamics during early sleep, serum TNF levels failed to show the full characteristics of a sleep regulatory substance (Krueger et al., 1999).

At a first glance, spontaneous TNF production measured in unstimulated monocytes seemed to mimic the observed changes in circulating TNF. However, unexpectedly the two measures did not correlate and sleep did not significantly suppress spontaneous monocytic TNF production at night. This could be taken to argue that sleep did not affect systemic TNF release from monocytes, but rather the release from other cellular sources such as tissue-resident macrophages (Zhou et al., 2001) or adipocytes (Schaffler et al., 2007). These cells are known to produce TNF upon adrenergic stimulation (Orban et al., 1999; Zhou et al., 2001) and in line with these findings our exploratory analyses indeed indicated a positive relationship between blood levels of norepinephrine and TNF.

In our experimental setting we carefully avoided sympathetic activation during the nocturnal vigil due to stress or physical activity which, in turn, can enhance TNF release (Bierhaus et al., 2003; Moldoveanu et al., 2000; Steptoe et al., 2007). Accordingly nighttime levels of epinephrine and norepinephrine showed only minor or no increments in the wake condition, respectively. On this background here we can only speculate about possible sympathetic contributions, i.e., that the lack of sleep at night might have induced subtle increases in sympathetic outflow to various tissues (Hornyak et al., 1991; Somers et al., 1993), which failed to induce a significant spill-over of norepinephrine into the circulation, but nevertheless might have sufficed to stimulate systemic TNF release.

Our findings of a relative decrease in circulating TNF levels accompanying nocturnal sleep in comparison with nocturnal sleep deprivation, as well as the relatively lower TNF levels during daytime after sleep deprivation, are not in line with other human studies, most of which failed to find any significant effect of acute sleep deprivation on nighttime or daytime levels of TNF (Irwin et al., 2004; Lekander et al., 2013; Ruiz et al., 2012; Shearer et al., 2001; Vgontzas et al., 2007). In addition, a recent study in healthy men showed an elevation of TNF levels in the evening after the nocturnal vigil (Chennaoui et al., 2011). These discrepancies might partly reflect confounding influences of the indwelling catheter which become evident after several hours (Haack et al., 2000). They might also be due to cytokine changes following heparin administration (Yamada et al., 1996), or reflect differences in the assays and experimental designs used in the cited studies (reviewed in Mullington et al., 2009). Of particular relevance is that recumbent and non-recumbent conditions specifically on the day after a nocturnal vigil are associated with opposite effects of sleep deprivation, i.e., decreases (Ogawa et al., 2003) and increases (e.g., (Zhong et al., 2005) in sympathetic outflow, respectively (reviewed in Mullington et al., 2009). In sum, based on the present data, there are several questions that need to be addressed in future studies, i.e., (i) how sleep deprivation relative to spontaneous nocturnal sleep mediates an acute enhancement in circulating TNF levels, (ii) whether there are counter regulatory mechanisms in healthy subjects that prevent circulating TNF levels from further increasing during the second half of the night or that might even decrease TNF levels the following day, and if so (iii) why these counter regulatory mechanisms might fail (Chennaoui et al., 2011) in particular in conditions of chronic sleep disturbance (Vgontzas et al., 2004). The latter aspect is of major clinical relevance, as it could explain the robust increases in blood levels of TNF during chronic sleep debt, e.g., in patients with sleep apnea, and corresponding metabolic and cardiovascular sequelae (Minoguchi et al., 2004).

4.2. LPS stimulated production of TNF by monocytes

As to LPS stimulated production of TNF, our results of a nighttime increase in stimulated TNF^+ monocytes agree with prior studies in humans on a regular sleep-wake cycle indicating a nocturnal peak of TNF production after in vivo LPS administration (Alamili et al., 2014), and in ex vivo LPS-stimulated blood cultures (Entzian et al., 1996; Hermann et al., 2006; Petrovsky et al., 1998; Zabel et al., 1993). This rhythm seems to relate to the strong circadian regulation of endotoxin sensitivity with peak mortality rates during the resting period, following experimental LPS injection in animals or in the course of septic conditions in patients (Halberg, 1963; Hrushesky and Wood, 1997; Marpegan et al., 2009; Scheff et al., 2010). Several studies revealed a direct contribution of clock genes to this temporal variation in stimulated TNF release (Keller et al., 2009; Nakao, 2014; Nguyen et al., 2013). Others focused on hormonal mediators and argued that the enhancing effect of prolactin (Cipollaro de et al., 1998; Dimitrov et al., 2004a) and the suppressing effects of cortisol (Hermann et al., 2006; Petrovsky et al., 1998; Petrovsky and Harrison, 1998; Waage and Bakke, 1988) and catecholamines (Dimitrov et al., 2013; Elenkov et al., 2008; Farmer and Pugin, 2000; Severn et al., 1992; van der Poll et al., 1994) on stimulated TNF production regulate the circadian rhythm in TNF release (DeRijk et al., 1997; Hermann et al., 2006; Meyer-Hermann et al., 2009; Scheff et al., 2010; Zoli et al., 2002). We likewise observed in our exploratory analyses that the percentage of LPS stimulated TNF^+ monocytes correlated positively with prolactin and negatively with cortisol and norepinephrine. Moreover, in our supplementary in vitro experiments adding cortisol, at concentrations typical for daytime wakefulness, to LPS stimulated whole blood cell cultures drawn during nocturnal sleep, reduced TNF^+ monocytes from 55% to 30%, which remarkably well mimics the morning decline in TNF^+ monocytes.

However, although the cortisol rhythm was preserved in conditions of continuous wakefulness, the rhythm in TNF^+ monocytes completely disappeared. We therefore posit that sleep, independent of cortisol, is a prerequisite for the rhythm in LPS-stimulated monocytic TNF production to occur. The sleep-dependent rise in prolactin was another likely candidate mechanism entraining TNF production to the sleep-wake rhythm. However contrary to this suspicion, blocking of prolactin in blood sampled during early nocturnal sleep failed to affect TNF^+ monocytes in our in vitro experiments. Thus, the mechanism promoting stimulated TNF production by monocytes during sleep remains unknown, and future studies might consider the possibility that several mediators conjointly act in an interactive or synergistic manner on monocytic TNF production.

Here we repeatedly measured stimulated TNF production on a single cell level in monocytes of undiluted whole blood cell cultures. This approach has several advantages, i.e., (i) not to miss sleep effects that are restricted to specific times of the day, (ii) to avoid confounding influences of changes in monocyte numbers in the cell culture, and (iii) not to decrease the effective concentrations of hormones or other active sleep-associated molecules, thus to avoid conditions which might have prevented the detection of an enhancing effect of sleep on LPS-stimulated TNF production in previous human experiments (Born et al., 1997; Spath-Schwalbe, 1992; Uthgenannt et al., 1995). In one study in mice, similar to the present findings in humans, a sleep-induced increase in stimulated TNF production after in vivo administration of LPS was observed that occurred in parallel in blood, spleen and brain, suggesting that this rise in TNF involves the whole organism (Weil et al., 2009). However, in two other experiments in vivo administration of LPS to humans (Haack et al., 2001) or Siberian hamsters (Ashley et al., 2013) failed to show such supporting effects of sleep on stimulated TNF production. Thus, it remains to be clarified

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whether our finding of sleep fostering stimulated TNF release in vitro, indeed, pertains to the in vivo situation.

Contrary to our results, two studies in humans (Irwin et al., 2006, 2010) revealed an increase in LPS-stimulated monocytic TNF production in the morning following sleep deprivation which was linked to activation of NF-κB, the key transcription factor in the inflammatory signaling cascade (Irwin et al., 2008). However, these findings cannot be directly compared with ours, because in these studies sleep deprivation was restricted to the first half of the night, and TNF production was measured only after subsequent recovery sleep.

The sleep-dependent rise in LPS-stimulated TNF production observed in the present study mirrors the supporting effects of sleep on LPS-stimulated IL-12 production by monocytes (Lange et al., 2006) and dendritic cell precursors (Dimitrov et al., 2007). In contrast, LPS-stimulated monocytic IL-6 production is not affected by sleep (Dimitrov et al., 2006), whereas LPS-stimulated production of the anti-inflammatory cytokine IL-10 by monocytes is suppressed during nocturnal sleep compared to continuous wakefulness (Lange et al., 2006). Thus, sleep exerts very distinct effects on different cytokines, which might be related to the finding that cytokines such as TNF and IL-6 show different sensitivities to hormonal regulation (DeRijk et al., 1997; Hermann et al., 2006; Szabo et al., 1997). Pathogen-triggered TNF release supports the very early stages of the development of adaptive immunity, namely migration, activation, and maturation of dendritic cells (Bauman et al., 2003; Caux et al., 1992; Trevejo et al., 2001). The increased stimulated production of TNF in the current study, and of IL-12, in previous experiments (Dimitrov et al., 2006; Lange et al., 2006) therefore offer an explanation for the promoting effect of sleep on the antigen-specific immune response to vaccinations that we (Lange et al., 2003, 2011) and others (Benedict et al., 2012; Prather et al., 2012; Spiegel et al., 2002) have previously reported. In this scenario, sleep and in particular SWS can be regarded as a host defense mechanism that is induced upon an infectious challenge, among others through the release of TNF, and that in turn promotes further TNF production (Toth, 1995).

Our findings on TNF measures have to be interpreted in light of some limitations. (i) Mainly due to the small sample size the results of our correlational analyses remain preliminary, and our study was not powered to analyze other conceivable influences on TNF measures, such as age, BMI (O'Connor et al., 2009) or the duration of certain sleep stages. (ii) Our study only included men; thus gender differences in the effects of sleep on TNF release observed in previous studies (Irwin et al., 2010; Mullington et al., 2009; Vgontzas et al., 2004), were not addressed. (iii) Finally, our results on sleep-induced increases in monocytic TNF production after stimulation with LPS might not pertain to other types of stimuli, e.g., phytohemagglutinin (Axelsson et al., 2013) or other cell types such as lymphocytes (Dimitrov et al., 2004b).

In conclusion, we report for the first time differential effects of sleep on unstimulated and stimulated TNF production in healthy men. While the acute suppression of circulating TNF levels during sleep indicates that sleep regulates spontaneous TNF release within hours, our finding of distinctly enhanced sleep-dependent stimulated TNF production adds to the notion that sleep provides an ideal environment to boost immune responses to an infectious challenge.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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